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Signal of the Prostate Specific Membrane Antigen

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<b>13. ABSTRACT (Maximum 200 Words)</b> The purpose of this award was to identify and characterize an internalization signal in the cytoplasmic tail of prostate specific membrane antigen (PSMA, transmembrane protein abundantly expressed in prostate cancer cells. These studies should aid in understanding the mechanism of antibody uptake and fate of the internalized antibody to improve antibody delivery approaches for immunotherapy for prostate cancer. We have now established and standardized a culture model to study internalization signal of PSMA. In addition we have determined that the cytoplasmic tail of PSMA has an internalization signal. An alanine scan mutagenesis approach in which each of the amino acids in the cytoplasmic tail is mutated to an alanine indicated that a di-leucine like motif in the cytoplasmic tail of PSMA might be involved in its internalization. Further mutagenesis approaches revealed that the N-terminal first five amino acids forms a novel endocytic determinant that render internalization function to a non-internalized protein. Thus we have now conclusively determined the endocytic determinant of PSMA.				
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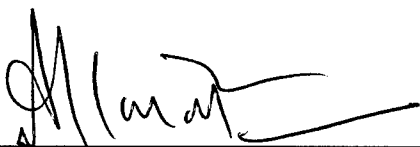
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## Introduction

PSMA, a transmembrane glycoprotein of approximately 100kD, is expressed almost exclusively in prostate epithelial cells. PSMA is a type II membrane protein with a short N-terminal cytoplasmic tail and a large C-terminal extracellular domain. Abundance of PSMA in prostate cancer cells and the cell surface localization of this protein make PSMA an ideal candidate for immunotherapy for prostate cancer. Novel PSMA-based prostate cancer therapies, including anti-PSMA monoclonal antibody (mAb)-based therapies are currently being investigated. An internalizing antibody is essential for improvement of immunotherapeutic approaches for prostate cancer. Understanding the mechanism of internalization of these antibodies is a crucial issue in prostate cancer research. Internalized antibody may be targeted to endosomes and recycled back to the cell surface or secreted out of the cell. Alternatively, the internalized antibody may be targeted to lysosomes via endosomes and degraded rapidly within lysosomes. In either case, the effectiveness of the antibody in immunotherapy is dramatically reduced. Prior knowledge of intracellular traffic of the internalized antibody should aid considerably in designing antibody delivery approaches for immunotherapy. Internalization and movement of proteins through endocytic pathway (i.e. targeting to endosomes or lysosomes) are mediated by internalization signal/s present in the cytoplasmic domain of internalized proteins. Anti-PSMA antibody internalization should be mediated by a specific amino acid sequence motif (internalization signal) of PSMA. Identification of the internalization signal of PSMA is crucial to understand the mechanism of antibody uptake and to determine the fate of internalized antibody. The purpose of this research is to identify internalization signal in the cytoplasmic tail that might be involved in targeting PSMA through the endocytic pathway.



## **Progress Report:**

### **Identification and characterization of an internalization signal of the Prostate Specific Membrane Antigen**

We have completed this project designed to determine the internalization signal of PSMA. In this report we show that the cytoplasmic tail of PSMA contains a leucine based internalization motif that mediates the internalization of this protein. We first identified that the two leucines in the cytoplasmic tail of PSMA is crucial for its internalization. We also determined that the first amino acid methionine is also important for the internalization of PSMA. Subsequently we have determined that the N-terminal five amino acids form an internalization motif that is transferable and renders internalization function to a non-internalized protein.

**Development of the culture model:** As indicated in my proposal our plan was to express a green fluorescent protein (GFP) tagged PSMA in LNCaP cells. To do this cDNA encoding full length PSMA was subcloned into EGFP vector (Clontech) and transfected into LNCaP cells by Lipofectamine method. Neomycin resistant cells expressing GFP were selected. During selection, these cells clearly showed fluorescence on the cell membrane as well as in intracellular vesicles as expected for PSMA localization in LNCaP cells. However, the fluorescence gradually diminished in culture and was not detectable for further studies by epifluorescence microscopy. Thus, we were unable to stably express PSMA-GFP fusion protein in LNCaP cells. However, when GFP was expressed alone it was clearly expressed in these cells. We further tested whether this problem is specific to PSMA, by tagging the  $\beta$ -subunit of sodium pump, a type II membrane protein like PSMA.  $\beta$ -subunit-GFP chimera behaved similarly in LNCaP cells indicating an inherent problem to express membrane protein-GFP chimera in LNCaP cells. Due to this problem we resorted to alternate methods to study the internalization signal of PSMA.

We selected COS (African green monkey kidney cells) that are extensively utilized for internalization studies of various proteins. The advantage of this cell line is that it can be transiently transfected with high transfection efficiency so that one can monitor internalization of proteins in transiently transfected cells. COS cells were transfected with full length PSMA cDNA in pCDNA3 vector. 48hrs after transfection, cells were incubated with monoclonal antibody against PSMA (mAb J591) for one hr. Cells were then fixed and permeabilized and stained with a fluorescent secondary antibody. This experiment clearly showed that mAb J591 was internalized in PSMA expressing cells. Double immunofluorescence localization using lysosomal and endosomal markers and laser scanning confocal microscopic visualization techniques were utilized to study the endocytic mechanisms of PSMA in COS cells. These results clearly revealed that PSMA is localized primarily in the endosomes with in 20 min of uptake and in 2 hrs is targeted to the lysosomes as we reported earlier in LNCaP cells (Liu et al., 1998, Cancer Res.58: 4055-4060). These results demonstrated that COS cells could be utilized as a model to characterize the internalization signal of PSMA.

First we determined that the cytoplasmic tail of PSMA contains an internalization signal. Then using alanine scan mutagenesis we have identified that a di-leucine motif in the cytoplasmic tail of PSMA might function as an internalization signal. To further confirm that the di-leucine motif of PSMA functions as the internalization signal and to identify the internalization motif we transferred the first five amino acids containing the di-leucine motif (LLDWM) to a non-internalized protein, the interleukin-2 receptor  $\alpha$ -chain (Tac). Tac is a transmembrane protein and like PSMA contains a small cytoplasmic tail. If the amino acid sequence LLDWM of PSMA is sufficient for PSMA internalization then the Tac containing the LLDWM amino acid sequence (Tac-LLDWM) in the cytoplasmic tail should internalize. Internalization of Tac was monitored by the uptake of a mAb 7G7, which is raised against the extracellular domain of Tac. In COS cells expressing a wild type Tac (Tac<sub>wt</sub>) construct, Tac was distinctly localized to the plasma membrane. As expected, incubation of these cells with mAb 7G7 did not result in the internalization of this antibody confirming that Tac<sub>wt</sub> is not internalized as reported earlier. By contrast, Tac-LLDWM internalized the mAb 7G7 and the internalized antibody clearly co-distributed with the internalized FITC-transferrin. These results indicated that the first five amino acids containing the di-leucine signal of PSMA is sufficient for its internalization and confers ability to internalize for a non-internalized protein. These results were submitted for publication (see enclosed manuscript). Based on the reviewers suggestions we further analyzed this internalization motif. We now determined that the first methionine and the fifth leucine of PSMA are crucial for its internalization. Also the length of the motif (five amino acids long) appears to be important since increasing the length of the motif by introducing an additional alanine inhibited the internalization. The revised manuscript is being written and will be submitted soon.

#### **Reportable outcomes:**

##### **Abstracts:**

Eri Oshima\*, Sigrid A Rajasekaran\*, Geoffrey Kim\*, Ning Ru\*, He Liu<sup>2</sup>, Warren Heston<sup>3</sup>, Neil H Bander<sup>2</sup>, Ayyappan K Rajasekaran (2001). A cytoplasmic tail di-leucine motif mediates the internalization and lysosomal targeting of prostate specific membrane antigen. (New discoveries in prostate cancer, AACR meeting, Naples, Florida).

##### **Manuscripts:**

1. Oshima E., Kim G., Rajasekaran S., Ru N., Liu H., Heston W., Bander N., Rajasekaran, A.K. A cytoplasmic tail di-leucine motif mediates the internalization and lysosomal targeting of prostate specific membrane antigen (submitted).
2. Krishnamachary B., Rajasekaran S., Liu H and Rajasekaran A.K. Differential requirements of cytoskeletal elements for the internalization of prostate specific membrane antigen in LNCaP cells and MDCK cells (in preparation).

**Conclusions:** We have identified that a leucine-based motif in the cytoplasmic tail of PSMA functions as an internalization and lysosomal targeting signal. Lysosomal targeting of PSMA indicates that the internalized PSMA antibodies will be targeted to the lysosomes and eventually be degraded. This might reduce the half-life of anti-PSMA antibodies used for the immunotherapy of prostate cancer. Therefore, these findings suggest that drugs that inhibit lysosomal targeting may improve the efficacy of anti-PSMA antibodies for treatment of prostate cancer.

**Key Research Accomplishments:**

- Identified an internalization signal in the cytoplasmic tail of PSMA.
- Identified and characterized that the N-terminal five amino acids forms an internalization motif of PSMA.
- Characterized that this motif is transferable and renders internalization function to a non-internalized protein.
- This is the first study, which showed that the translation start site of a protein is also associated with the internalization function of a protein.

A cytoplasmic tail di-leucine motif mediates the internalization and lysosomal targeting of prostate specific membrane antigen

Eri Oshima\*, Sigrid A Rajasekaran\*, Geoffrey Kim\*, Ning Ru\*, He Liu\*\*, Warren Heston\*\*\*, Neil H Bander\*\*, Ayyappan K Rajasekaran\* \*Department of Pathology and Laboratory Medicine, University of California, Los Angeles, Los Angeles, CA 90095, \*\*Department of Urology, Weill Medical College of Cornell University, New York, NY 10021, \*\*\*Department of Cancer Biology, Cleveland Clinic Foundation, Cleveland, OH, 44195.

Prostate Specific Membrane Antigen (PSMA) is a transmembrane protein expressed at high levels in prostate cancer and in tumor associated neovasculature. We have shown that PSMA undergoes internalization via clathrin-coated vesicles in prostate cancer cells (Liu et al., Cancer Res. 58: 4055-4060, 1998). In this study, we report that the internalization of PSMA is mediated by a di-leucine motif present in its cytoplasmic tail. Cytoplasmic tail mutants of PSMA were transiently expressed in COS cells and internalization was monitored by uptake of an extracellular domain specific monoclonal antibody (mAb J591) and laser scanning confocal microscopy. Deletion of the cytoplasmic tail or mutating the di-leucine pair (amino acid residues 4 and 5) to di-alanine resulted in the loss of internalization of mAb J591. While mutation of amino acid residue 4 to alanine did not affect internalization, mutation of amino acid residue 5 to alanine strongly inhibited the uptake of mAb J591. Furthermore, using a chimeric protein (Tac- LLNWM) composed of the Tac antigen, the  $\alpha$ -chain of interleukin 2-receptor, fused to the pentapeptide (LLNWM) of PSMA we found that this sequence is sufficient for PSMA internalization and lysosomal targeting. We suggest that the di-leucine motif of PSMA directs receptor mediated endocytosis and lysosomal targeting of PSMA.

01 OCT 2001

**A cytoplasmic tail di-leucine motif mediates the internalization and lysosomal targeting of prostate specific membrane antigen**

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**Running Title:** Internalization signal of PSMA

**Key Words:** Prostate Specific Membrane Antigen, Endocytosis, Di-leucine motif, Internalization signal, Lysosomal targeting signal.

**Abbreviations:** PSMA, prostate specific membrane antigen, NAALDase, N-acetylated  $\alpha$ -linked acidic dipeptidase.

## Abstract

Prostate Specific Membrane Antigen (PSMA) is a transmembrane protein expressed at high levels in prostate cancer and in tumor associated neovasculature. We have shown that PSMA undergoes internalization via clathrin-coated vesicles in prostate cancer cells (Liu et al., Cancer Res. 58: 4055-4060, 1998). In this study, we report that the internalization of PSMA is mediated by a di-leucine motif present in its cytoplasmic tail. Cytoplasmic tail mutants of PSMA were transiently expressed in COS cells and internalization was monitored by uptake of an extracellular domain specific monoclonal antibody (mAb J591) and laser scanning confocal microscopy. Deletion of the cytoplasmic tail or mutating the di-leucine pair (amino acid residues 4 and 5) to di-alanine resulted in the loss of internalization of mAb J591. While mutation of amino acid residue 4 to alanine did not affect internalization, mutation of amino acid residue 5 to alanine strongly inhibited the uptake of mAb J591. Furthermore, using a chimeric protein (Tac-LLNWM) composed of the Tac antigen, the  $\alpha$ -chain of interleukin 2-receptor, fused to the pentapeptide (LLNWM) of PSMA we found that this sequence is sufficient for PSMA internalization and lysosomal targeting. We suggest that the di-leucine motif of PSMA directs receptor mediated endocytosis and lysosomal targeting of PSMA.

## Introduction

Prostate Specific Membrane Antigen (PSMA) was originally identified by the monoclonal antibody 7E11-C5 raised against the human prostate cancer cell line LNCaP (Horoszewicz et al., 1987). Subsequently, the PSMA gene was cloned (Israeli, 1993), and mapped to chromosome 11q14 (Rinker-Schaeffer et al., 1995). PSMA is a type II membrane protein with a short cytoplasmic N-terminal region (19 amino acids) a transmembrane domain (24 amino acids) and a large extracellular C-terminal portion (707 amino acids) (Israeli et al., 1993). The extracellular domain of PSMA has several potential N-glycosylation sites and shows homology (26% identity at the amino acid level) to the transferrin receptor I (Israeli et al., 1993) and a recently cloned transferrin receptor II (Kawabata et al., 1999). The significance of PSMA homology to the transferrin receptor is not known.

PSMA has been the subject of increasing interest in cancer research due to its potential as a diagnostic and therapeutic target for human prostate cancer (Chang et al., 1999a). PSMA is abundantly expressed in prostate cancer cells. Its expression is further increased in higher-grade cancers, metastatic disease, and hormone-refractory prostate carcinoma (Silver et al., 1997; Wright et al., 1996). In addition, PSMA has become the focus of even more intense interest due to the recent findings that it is selectively expressed in the neovasculature of nearly all types of solid tumors, but not in the vasculature of normal tissue (Chang et al., 1999b; Chang et al., 1999c; Liu et al., 1997; Silver et al., 1997). The function of PSMA with respect to vascular endothelial cell biology and the direct correlation between its expression and increasing tumor aggressiveness in prostate cancer remain intriguing and unclear. Recently, it has been shown that PSMA is homologous to glutamate carboxypeptidase II (85% at nucleic acid level) isolated from rat brain (Coyle, 1997), has folate hydrolase activity (Halsted et al., 1998; Pinto et al., 1996) and

N-acetylated  $\alpha$ -linked acidic dipeptidase (NAALDase) activity (Carter et al., 1996; Luthi-Carter et al., 1998). Although a significant amount of research is being carried out on PSMA as a diagnostic marker and a therapeutic target for prostate cancer and on enzymatic activities tissues, very little is known about the cell biology of this protein.

Monoclonal antibodies against extracellular domain of PSMA hold potential in diagnostic tumor imaging and as therapeutic agents (Chang et al., 1999a). Internalizing antibodies are required when toxins, drugs or short-range isotopes are to be delivered specifically to the interior of cancer cells. The formation of antigen-antibody complexes on the cell surface often results in internalization through the pathway closely resembling receptor-mediated endocytosis of peptide hormones, growth factors and other natural ligands (Pastan and Willingham, 1981). In general, the endocytic pathway includes internalization of the receptor-ligand complex via clathrin-coated pits and accumulation in the endosomes. The receptor-ligand complex then dissociates in the endosomes and the dissociated molecules are either recycled back to the cell surface or targeted to lysosomes for degradation (Mukherjee et al., 1997). Targeting of most receptors to coated pits and their traffic through endocytic compartments are generally mediated by a specific internalization motif in the cytoplasmic domain of proteins (Schmid, 1992). The first well characterized internalization motif of transferrin receptor, mannose-6-phosphate receptor, asialoglycoprotein receptor, polymeric immunoglobulin receptor and others are all tetrapeptides (Tyr-X-Arg-Phe) having an aromatic residue in the fourth position of the sequence (Bonifacino and Dell'Angelica, 1999; Trowbridge, 1991). The tyrosine residue of the internalization motif appears to be crucial for the internalization of proteins (Ktistakis et al., 1990). The second type of signal is a so-called di-leucine based (LL) signal, for which the only known requirement is the presence of two consecutive leucines or a leucine-isoleucine pair. LL-based signals have been



shown to mediate internalization and targeting to endosomes and lysosomes (Kil et al., 1999; Letourneur and Klausner, 1992). Di-leucine or leucine-isoleucine signals have been identified in lysosomal proteins such as mannose 6-phosphate/insulin-like growth factor-II receptor (Johnson and Kornfeld, 1992a), the cation-dependent mannose-6-phosphate receptor (Johnson and Kornfeld, 1992b), and lysosomal integral membrane protein II (Vega et al., 1991).

We have shown that PSMA undergoes internalization via clathrin-dependent endocytosis in LNCaP cells (Liu et al., 1998) a human prostate cancer cell line that abundantly express endogenous PSMA. To further understand the mechanism of PSMA internalization we searched for possible internalization motifs in the cytoplasmic domain of PSMA. In this study we demonstrate that the di-leucine motif in the cytoplasmic tail of PSMA mediates its internalization and lysosomal targeting. By transferring the di-leucine motif to a non-internalized protein, the  $\alpha$ -chain of interleukin 2-receptor (Tac) we further show that the di-leucine signal of PSMA is sufficient for its internalization and targeting to lysosomes.

## **Experimental procedures**

### **Plasmid constructs**

Cloning and characterization of full-length cDNA of PSMA was described earlier (Israeli et al., 1993). The alanine scan mutagenesis approach was utilized to mutate each of the cytoplasmic tail amino acids in the cytoplasmic tail of PSMA. Alanine scan mutagenesis was essentially carried out by polymerase chain reaction (PCR) using sense primers carrying respective mutation of the cytoplasmic tail amino acid (positions 2-15) to alanine. A Kozak consensus sequence (GCCACC) and a translation start site (ATG) were incorporated at the N-terminus of the sense primers. An antisense primer at the 3' end of the PSMA was utilized to create point mutations in the cytoplasmic tail of PSMA. A cytoplasmic deletion mutant of PSMA was created by deleting the N-terminal 15 amino acids using polymerase chain reaction (PCR). An alanine and three arginine residues proximal to the transmembrane were retained since the three arginine residues proximal to the transmembrane proteins may be necessary to maintain the type II orientation of the protein (von Heijne, 1988). In addition to the point mutations the putative di-leucine pair (positions 4 and 5) was converted to alanine using a similar strategy as described above. Also a PSMA construct in which the cytoplasmic tail amino acids 6-14 were deleted and all the three putative phosphorylation sites mutated (PSMA-T8A/S10A/T14A) were generated using PCR. Four Tac-PSMA chimeras were also generated using PCR. Full-length Tac (gift from Dr. Bonifacino, National Institute of Health, MD) was described earlier (Leonard et al., 1984). Tac cytoplasmic tail containing the di-leucine motif of PSMA (Tac-LLNWM), di-leucine motif mutated to alanine (Tac-AANWM), leucine at position 5 mutated to alanine (Tac-ALNWM), and leucine at position 4 mutated to alanine (Tac-LANWM) were generated using primers carrying the respective mutations. Full-length PSMA (designated as wild type PSMA

(PSMA<sub>wt</sub>)), cytoplasmic tail mutants of PSMA, and Tac-PSMA chimeras were inserted into eukaryotic expression vector pcDNA3. The mutations were verified by DNA sequencing.

Constructs utilized in this study are shown in figure 1.

### **Cell culture and transfection**

COS-7 cells (ATCC CRL 1651) were grown in DMEM at 5% CO<sub>2</sub> in a water-saturated atmosphere. All culture media were supplemented with 10% fetal bovine serum containing streptomycin and penicillin. Cells grown on glass coverslips were transiently transfected by the calcium phosphate method as described earlier (Rajasekaran et al., 1994). After transfection (48hours) the cells were tested for the uptake of antibodies as described below.

### **Antibody uptake and immunofluorescence analysis**

Antibody uptake was carried out as described earlier (Liu et al., 1998). In brief, the cells were washed with DMEM containing 0.5% fatty acid free BSA (DMEM-BSA) and incubated at 37°C for 2 hours with mAb (J591, 5µg/ml). Cells were then fixed, permeabilized and incubated with Texas-Red conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). To visualize PSMA localization in endosomes, cells were co-incubated with FITC conjugated transferrin (Jackson ImmunoResearch, Westgrove, PA) during J591 incubation. To monitor the internalization of Tac-PSMA chimeras, monoclonal antibody against the extracellular domain of Tac, 7G7 (Rubin et al., 1985) was utilized. Uptake of antibodies (mAbs J591 and 7G7) and transferrin were visualized by confocal microscopy. To visualize surface expression of the PSMA and Tac-PSMA chimeras, COS cells transfected with the respective plasmid were fixed and stained with mAb J591 under non-permeabilized conditions. Lysosomes were visualized using antibodies against a lysosomal marker Lamp -I (gift from Dr. Minoru Fukuda, Burnham

Institute, La Jolla, CA). Surface expression and co-distribution of mAb J591 with lysosomes were determined by confocal microscopy.

### **Confocal microscopy**

Co-distribution of internalized mAbs J591 or 7G7 and transferrin or Lamp-1 were examined using a Fluoview laser scanning confocal microscope (Olympus America Inc, Melville, NY). To detect simultaneously FITC- and Texas red-labeled antigens, samples were excited at 488 and 568 nm with Argon and Krypton lasers, respectively, and the light emitted between 525-540 nm was recorded for FITC and above 630 nm for Texas red. Images were generated using Fluoview software (version 2.1.39). 30-40 transfected cells were examined for each transfection done on duplicate cover slips and the representative data are shown.

### **NAALDase activity**

NAALDase activity was determined as described by (Sekiguchi et al., 1989). COS cells were transfected with PSMA<sub>wt</sub> on 60mm culture dishes. After 48 hours of transfection, cells were incubated with 1  $\mu$ Ci/ml <sup>3</sup>H-NAAG (NEN, Boston, MA) in Krebs-Ringer bicarbonate medium or in Dulbecco's modified eagle medium for 1 hour. The medium was removed and the cells were washed three times with their respective incubation medium. Cells were then lysed in 1% Triton-X-100 and the radioactivity was determined using a scintillation counter (Beckman LS 6500). Counts were normalized to protein. Protein concentrations of the cell lysates were determined using the BioRad DC reagent (BioRad Laboratories, Hercules, CA) according to manufacturer's instructions.

## Results

Since LNCaP cells express abundant amounts of endogenous PSMA, these cells were not ideal for experiments designed to identify the internalization signal of PSMA. Therefore, we utilized COS cells that do not express endogenous PSMA. To identify the internalization signal, we made deletions and mutations in the cytoplasmic tail of PSMA and expressed these mutants transiently in COS cells (Fig. 1). The internalization of PSMA was examined by the uptake of mAb J591. Confocal microscope X-Y optical sections of cells double-labeled with transferrin (early endosomal marker) and PSMA were utilized to confirm the internalization of PSMA in COS cells expressing various deletion and point mutants of PSMA.

Transient transfection of PSMA<sub>wt</sub> in COS cells resulted in its plasma membrane expression as revealed by immunofluorescence staining under non-permeabilized condition (Fig. 2A). Incubation of these cells with mAb J591 resulted in the internalization of this antibody (Fig. 2B) and its accumulation in endosomes as revealed by its co-distribution with internalized transferrin (Fig. 2 C and D). Most of the surface localized PSMA appeared to be internalized since only sparse plasma membrane staining was detected in these cells as compared to non-permeabilized cells (Fig. 2, compare panel A and B). Untransfected cells did not show internalization of mAb J591 yet clearly showed the uptake of FITC-transferrin (data not shown). These results indicate that in COS cells, PSMA is internalized and is localized to endosomes as reported previously in LNCaP cells as (Liu et al., 1998).

We next examined whether the cytoplasmic tail of PSMA contains a signal that mediates its internalization. For this purpose the cytoplasmic tail of PSMA was deleted and the deletion mutant (PSMA- $\Delta$ CD) was expressed in COS cells. PSMA- $\Delta$ CD was clearly expressed on the cell surface as revealed by immunofluorescence staining under non-permeabilized condition (Fig.

2E). Incubation of these cells with mAb J591 did not show uptake or localization to the endosomes as internalized transferrin did not reveal a co-distribution with PSMA (Fig. 2F, G and H). This result indicated that the cytoplasmic tail of PSMA contains a signal that mediates its internalization.

To examine whether the di-leucine motif of PSMA's cytoplasmic tail functions as an internalization signal, the di-leucine motif was converted to di-alanine (PSMA-L4A/L5A), the mutant protein was expressed in COS cells and the uptake of mAb J591 was monitored. As shown in figure 3, the di-alanine mutant of PSMA was clearly expressed on the cell surface as revealed by staining with mAb J591 under non-permeabilized condition (Fig. 3A). Our internalization assay revealed that mAb J591 was not internalized in cells expressing the di-alanine mutant of PSMA (Fig. 3B) and did not show co-distribution with the internalized FITC-transferrin (Fig. 3C and D) indicating that mutation of the di-leucine motif in the cytoplasmic tail of PSMA abrogates its internalization. We then examined whether both these leucines are essential for the internalization of PSMA. For this purpose leucine residues at positions 4 (PSMA-L4A) and 5 (PSMA-L5A) were mutated to alanine and the uptake of mAb J591 was studied. As shown in figure 3, both these mutants were clearly expressed on the cell surface as revealed by staining with mAb J591 under non-permeabilized condition (Fig. 3E and I). The mAb J591 was clearly internalized in cells expressing PSMA-L4A (Fig. 3F). In these cells the internalized transferrin (Fig. 3G) clearly co-distributed with the internalized mAb J591 (Fig. 3H). By contrast, in cells expressing PSMA-L5A, mAb J591 was not internalized (Fig. 3J) and the antibody primarily stained the plasma membrane as seen in cells expressing PSMA-L4/L5 (Fig. 3B). In these cells distinct co-localization of mAbJ591 staining and internalized FITC-transferrin was not detected (Fig. 3K and L). These results indicated that the fifth leucine is crucial for the

internalization of PSMA and is less resilient to changes. To further test whether amino acid residues other than the di-leucine motif are essential for the internalization of mAb J591 we systematically mutated each of the cytoplasmic tail amino acids into alanine. These point mutations did not affect the internalization of mAb J591 (data not shown). Moreover, the construct in which amino acids 6-14 were deleted (PSMA- $\Delta$ 6-14cd) when expressed in COS cells internalized mAb J591. Taken together, these results indicated that the di-leucine motif mediates the internalization of PSMA.

To further confirm that the di-leucine motif of PSMA is sufficient for the internalization of PSMA, we transferred the first five amino acids of PSMA containing the di-leucine motif to the non-internalized protein Tac (Letourneur and Klausner, 1992). If the di-leucine motif is sufficient for PSMA internalization, addition of this motif to the cytoplasmic tail of Tac should induce internalization of Tac. Internalization of Tac was monitored by uptake of mAb 7G7, raised against the extracellular domain of Tac (Rubin et al., 1985). In non-permeabilized COS cells wild type Tac (Tac<sub>wt</sub>) construct showed distinct plasma membrane localization (Fig. 4A) indicating that this protein is targeted to the plasma membrane in COS cells. As expected, incubation of these cells with mAb 7G7 did not result in the internalization of this antibody as reported earlier (Letourneur and Klausner, 1992), confirming that Tac<sub>wt</sub> is not internalized (Fig. 4B). Co-distribution of mAb 7G7 staining and internalized transferrin was not detected in these cells (Fig. 4C and D). By contrast, incorporation of the amino acids LLNWM to the Tac cytoplasmic tail (Tac-LLNWM) resulted in the internalization of mAb 7G7 (Fig. 4F) and the internalized antibody clearly co-localized with the internalized FITC-transferrin (Fig. 4G and H) indicating that the di-leucine motif in the cytoplasmic tail of PSMA is transferable and is sufficient to confer internalization to a non-internalized protein.

Furthermore, in cells expressing Tac-AANWM where the di-leucine motif is mutated to di-alanine, the mAb 7G7 was not internalized (Fig. 5B) although this protein was clearly localized to the plasma membrane (Fig. 5A). This mutant did not co-distribute with the internalized FITC-transferrin (Fig. 5 C and D). Internalization of mAb 7G7 was maintained in cells expressing the construct where the fourth leucine is mutated to alanine (Tac-LANWM) (Fig. F, G and H) whereas cells expressing Tac-ALNWM, where the leucine at position 5 is mutated, uptake of 7G7 was not detected (Fig. 5 J, K and L). Both these mutants were clearly expressed on the plasma membrane as revealed by non-permeabilized staining using mAb 7G7 (Fig. 5E and I). Taken together, these data demonstrate that the di-leucine motif of PSMA functions as an internalization signal and that the leucine at the fifth position is more critical for internalization.

A cytoplasmic di-leucine motif has been shown to be involved in the lysosomal targeting of several membrane proteins (Calvo et al., 1999; Dittrich et al., 1996; Haft et al., 1994; Kil et al., 1999; Letourneur and Klausner, 1992). Therefore, we tested whether the di-leucine motif of PSMA also functions as a lysosomal targeting signal. For this purpose, COS cells transfected with PSMA<sub>wt</sub> and PSMA-L4A/L5A, were incubated with mAb J591. Cells were then immunofluorescently double-labeled to detect internalized mAb J591 and a lysosomal marker Lamp-1 (Fukuda et al., 1988) and visualized by confocal microscopy. As shown in figure 6, cells expressing PSMA<sub>wt</sub> showed co-localization with LAMP-1 containing lysosomal vesicles (Fig. 6A-C). In contrast, cells expressing the PSMA-L4A/L5A construct showed PSMA staining predominantly on the plasma membrane and no lysosomal staining was detected (Fig. 6D-F). Cells expressing Tac-LLNWM showed co-localization of Tac and Lamp-1 (Fig. 6G-I) where as in cells expressing Tac-AANWM this co-localization was not detected (Fig. 6J-L). From these



results we conclude that the di-leucine motif of PSMA functions not only as internalization signal but also mediates its targeting to lysosomes.

## Discussion

A large body of evidence has accumulated linking di-leucine signals located within the cytoplasmic tail of transmembrane proteins to internalization, lysosomal targeting and sorting of these proteins at the level of the trans-Golgi network (Kirchhausen et al., 1997; Trowbridge et al., 1993). In this study, we demonstrated that the di-leucine motif in the cytoplasmic tail of PSMA functions as an internalization and lysosomal targeting signal. We showed that addition of the pentapeptide LLNWM to the cytoplasmic tail of Tac antigen enables this molecule to be internalized and targeted to the lysosomes indicating that the pentapeptide motif is sufficient for PSMA internalization and lysosomal targeting. The PSMA cytoplasmic tail deletion mutant (PSMA-ΔCD) as well as mutants that failed to internalize (PSMA-L4A/L5A, PSMA-L5A, Tac-AANWM, Tac-ALNWM) were expressed on the plasma membrane. This result indicates that lack of internalization of these mutants is not due to their loss of cell surface expression and suggests that the cytoplasmic tail deletion or mutations have less impact on the transport of these mutant proteins to the cell surface while they largely inhibit their ability to internalize.

In general, one of the leucines of the di-leucine motif is more critical for internalization and appears less resilient to changes. In the CD3-γ chain, mutation of the second leucine (L131) still internalizes whereas mutation of the first leucine (L130) abolishes internalization (Letourneur and Klausner, 1992). In gp130, a signal transducing component associated with the interleukin-6 receptor, the second leucine can be changed without loss of internalization whereas mutation of the first leucine abolished its internalization function (Dittrich et al., 1996). In PSMA, mutation of the first leucine (Leu-4) did not change significantly the internalization of mAb J591. In contrast, conversion of the second leucine (Leu-5) resulted in the complete loss of

internalization indicating that this leucine is less resilient to change and is more critical for the internalization of PSMA.

Recent studies indicate that the di-leucine motif associates with adaptor complexes AP1 and AP2 (Hofmann et al., 1999; Rapoport et al., 1997). Binding of a leucine-based sorting signal to AP-3 has been shown for lysosomal membrane protein LIMP-II and the melanosomal membrane protein tyrosinase (Höning et al., 1996). It is possible that these adaptors might bind to the cytoplasmic tail of PSMA and might be involved in PSMA internalization and lysosomal targeting. Experiments are in progress in our laboratory to identify the binding partners of the cytoplasmic tail of PSMA that might be involved in its internalization.

Amino acids proximal to the di-leucine signal have been shown to modulate the function of the di-leucine signal. The di-leucine signal of CD4 is active only when adjacent serine residues are phosphorylated (Pitcher et al., 1999). In gp130, mutation of a serine residue located 6 amino acids upstream of the di-leucine signal has been shown to be important for rapid internalization of IL-6 (Dittrich et al., 1996). The cytoplasmic tail of PSMA has a consensus protein kinase C sequence (position 14 (Thr)) and two other hydroxyl-containing residues (positions 8 (Thr) and 10 (Ser)) that might serve as phosphorylation acceptor sites, and it has been suggested that phosphorylation mechanisms may regulate PSMA function (Luthi-Carter et al., 1998). However, whether PSMA is phosphorylated or the significance of PSMA phosphorylation is currently not known. Mutation of the individual phosphorylation sites or mutation of all the three-phosphorylation sites did not reveal a visible effect on the internalization of mAb J591 as revealed by immunofluorescence analysis (data not shown). Furthermore, our alanine scan mutagenesis in which each of the amino acids in the cytoplasmic tail of PSMA was mutated to alanine revealed that mutation of amino acid residues other than the

critical 5<sup>th</sup> leucine residue did not significantly affect the internalization of PSMA indicating that these amino acids probably play little, if any, role in the internalization of PSMA.

The catalytic site for glutamate carboxypeptidase/NAALDase activity of PSMA resides in the extracellular domain of this protein (Speno et al., 1999). Millimolar concentration of phosphate used in the culture medium almost completely inhibited the NAALDase activity in COS cells (data not shown; (Slusher et al., 1999) and this activity appears not to be necessary for the internalization of PSMA. Moreover, in LNCaP cells, incubation with the NAAG substrate for NAALDase did not increase the internalization of PSMA (data not shown) whereas incubation with mAb J591 increased the internalization of PSMA (Liu et al., 1998). These results indicate that the internalization of PSMA might be an independent function from its glutamate carboxipeptidase/NAALDase activity.

It has been shown that antibodies may exert an inhibitory effect on cancer cells via antibody-induced removal of an oncoprotein from the cell surface (Fan and Mendelsohn, 1998). For example, the oncogenic potential of ErbB-2 depends on its localization to the plasma membrane (Beerli et al., 1994; Flanagan and Leder, 1988) and antibodies that remove ErbB molecules from the cell surface and direct them to the endocytic pathway are excellent cancer inhibitors (Hurwitz et al., 1995). Increased expression of PSMA in high grade and metastatic prostate cancers and its expression in tumor vasculature strongly suggest that PSMA function might be associated with the oncogenic process. It is possible that the plasma membrane localization of PSMA might be involved in its putative role in cancer. Our results suggest that the di-leucine motif of PSMA is involved in the targeting of PSMA to the lysosomes and might negatively regulate the levels of PSMA protein in cells. It is tempting to speculate that di-leucine based internalization and lysosomal targeting by potentially therapeutic antibodies against PSMA

might be involved in the clearance of PSMA from the cancer cell surface and might reduce the putative role of PSMA in the oncogenic process.

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## Figure Legends:

**Figure 1. Schematic representation of PSMA cytoplasmic tail mutants and Tac-PSMA chimera used in this study.** Deletions are shown by horizontal arrows. Amino acids converted to alanine or valine are indicated as A and V, respectively. The internalization (INT) positive (+) or negative (-) for the respective constructs are shown.

**Figure 2. Analysis of the internalization of PSMA in COS-7 cells expressing wild type PSMA (PSMA<sub>wt</sub>) and the cytoplasmic tail deletion mutant (PSMA- $\Delta$ cd).** To visualize surface expression of PSMA, 48 h after transfection cells were fixed in paraformaldehyde under non-permeabilized condition and labeled with mAb J591 followed by FITC-conjugated anti-mouse antibody and visualized by epifluorescence microscopy (Panels A and E). To monitor internalization of PSMA, cells were incubated with mAb J591 and FITC-transferrin for 2 h. Cells were washed, fixed in cold methanol and stained with Texas-red conjugated anti-mouse antibody. Representative medial optical sections are shown. Panels B, F and C, G represent labeling of PSMA and transferrin, respectively. Panels D and H are merged images. Yellow color in D indicates the co-distribution of FITC-transferrin and internalized PSMA. Bars =10 $\mu$  (A, E) and 5 $\mu$  (B, C, D, F, G, and H).

**Figure 3. Analysis of internalization of the cytoplasmic tail di-leucine mutants of PSMA.** Surface expression as well as internalization of PSMA was performed as described in figure legend 2. Panels A, E and I represent surface expression of PSMA in COS-7 cells expressing PSMA-L4A/L5A, PSMA L4A and PSMA L5A mutants, respectively. Panels B, F, J and C, G, K represents labeling of PSMA and transferrin, respectively. Panels D, H, and L

represent merged images. Representative medial optical sections are shown. Yellow color in H indicates the co-distribution of FITC-transferrin and internalized PSMA. Bars =10 $\mu$  (A, E, and I) and 5 $\mu$  (B, C, D, F, G, H and J, K, L).

**Figure 4. Analysis of the internalization of Tac and Tac-PSMA chimeras.** To visualize surface expression of Tac, 48 h after transfection cells were fixed in paraformaldehyde under non-permeabilized condition and labeled with mAb 7G7 followed by FITC-conjugated anti-mouse antibody and visualized by epifluorescence microscopy (Panels A and E). To monitor internalization of Tac, cells were incubated with mAb 7G7 and FITC-transferrin for 2 h. Cells were washed, fixed in cold methanol and stained with Texas-red conjugated anti-mouse antibody. Representative medial optical sections are shown. Panels B, F and C, G represent labeling of Tac and transferrin, respectively. Panels D and H are merged images. Yellow color in H indicates the co-distribution of FITC-transferrin and internalized Tac. Bars =10 $\mu$  (A, E) and 5 $\mu$  (B, C, D, F, G, and H).

**Figure 5. Analysis of internalization of the Tac-PSMA chimeras harboring mutations in the di-leucine signal.** Surface expression as well as internalization of PSMA was performed as described in figure legend 4. Panels A, E and I represent surface expression of Tac in COS-7 cells expressing Tac-AANWM, Tac-LANWM, and Tac-ALNWM chimeras, respectively. Panels B, F, J and C, G, K represents labeling of Tac and transferrin, respectively. Panels D, H, and L represent merged images. Representative medial optical sections are shown. Yellow color in H indicates the co-distribution of FITC-transferrin and internalized PSMA. Bars =10 $\mu$  (A, E and I) and 5 $\mu$  (B, C, D, F, G, H and J, K, L).

**Figure 6. Localization of PSMA<sub>wt</sub>, PSMA-L4A/L5A, Tac-LLNWM, and Tac-**

**AANWM relative to the lysosomal marker Lamp-1.** 48 h after transfection COS-7 cells were incubated for 2 h with mAb J591 (A-F) or mAb 7G7 (G-L). Cells were fixed, permeabilized, and stained with mAb J591 (A,D) or mAb 7G7 (G, J) and rabbit antiserum against Lamp-1 (B, E, H and K). Panels C, F, I, and L represent merged images. Yellow color in panels C and I indicate co-distribution of PSMA and Tac with Lamp-1. Bar = 5 $\mu$ .



# Figure 1

Construct	Cytoplasmic Tail Sequence																	Int		
<b>PSMA</b>																				
Wt	M	W	N	L	L	H	E	T	D	S	A	V	A	T	A	R	R	P	R	+
Δcd	M	→													A	R	R	P	R	-
Ala 4,5	-	-	-	A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ala 4	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Ala 5	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ala 2	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Ala 3	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Ala 6	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Ala 7	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	+
Ala 8	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	+
Ala 9	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	+
Ala 10	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	+
Val 14	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	+
Ala 8,10,14	-	-	-	-	-	-	-	A	-	A	-	-	-	A	-	-	-	-	-	+
Δ6-14	M	W	N	L	L	→									A	R	R	P	R	+
<b>Tac</b>																				
Tac Fl	R	R	Q	R	K	S	R	R	T	I									-	
Tac Di-Leu	-	-	-	-	-	-	-	-	-	-	L	L	N	W	M					+
Tac Di-Ala	-	-	-	-	-	-	-	-	-	-	A	A	N	W	M					-
Tac Ala 4	-	-	-	-	-	-	-	-	-	-	L	A	N	W	M					+
Tac Ala 5	-	-	-	-	-	-	-	-	-	-	A	L	N	W	M					-

Figure 2

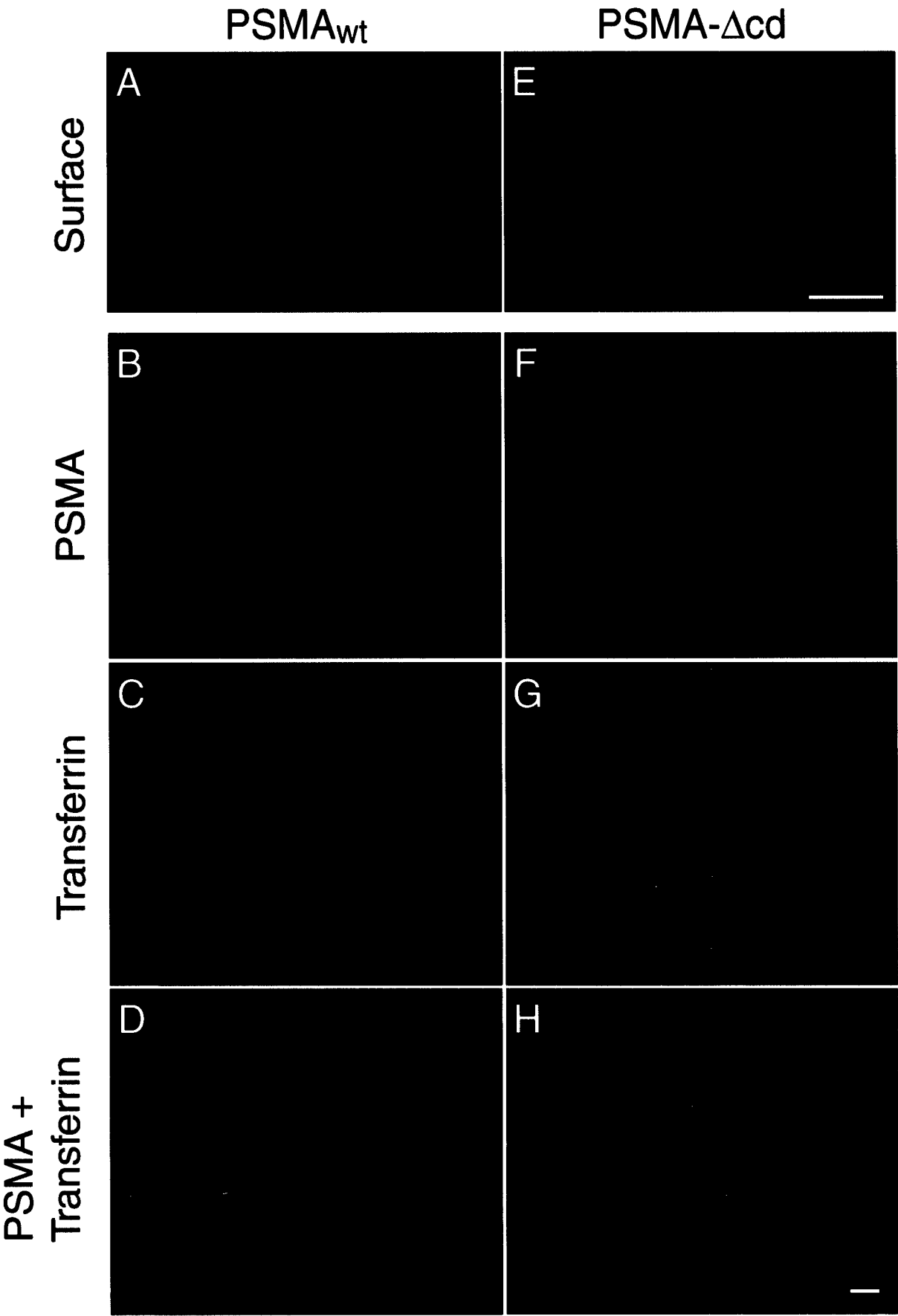


Figure 3

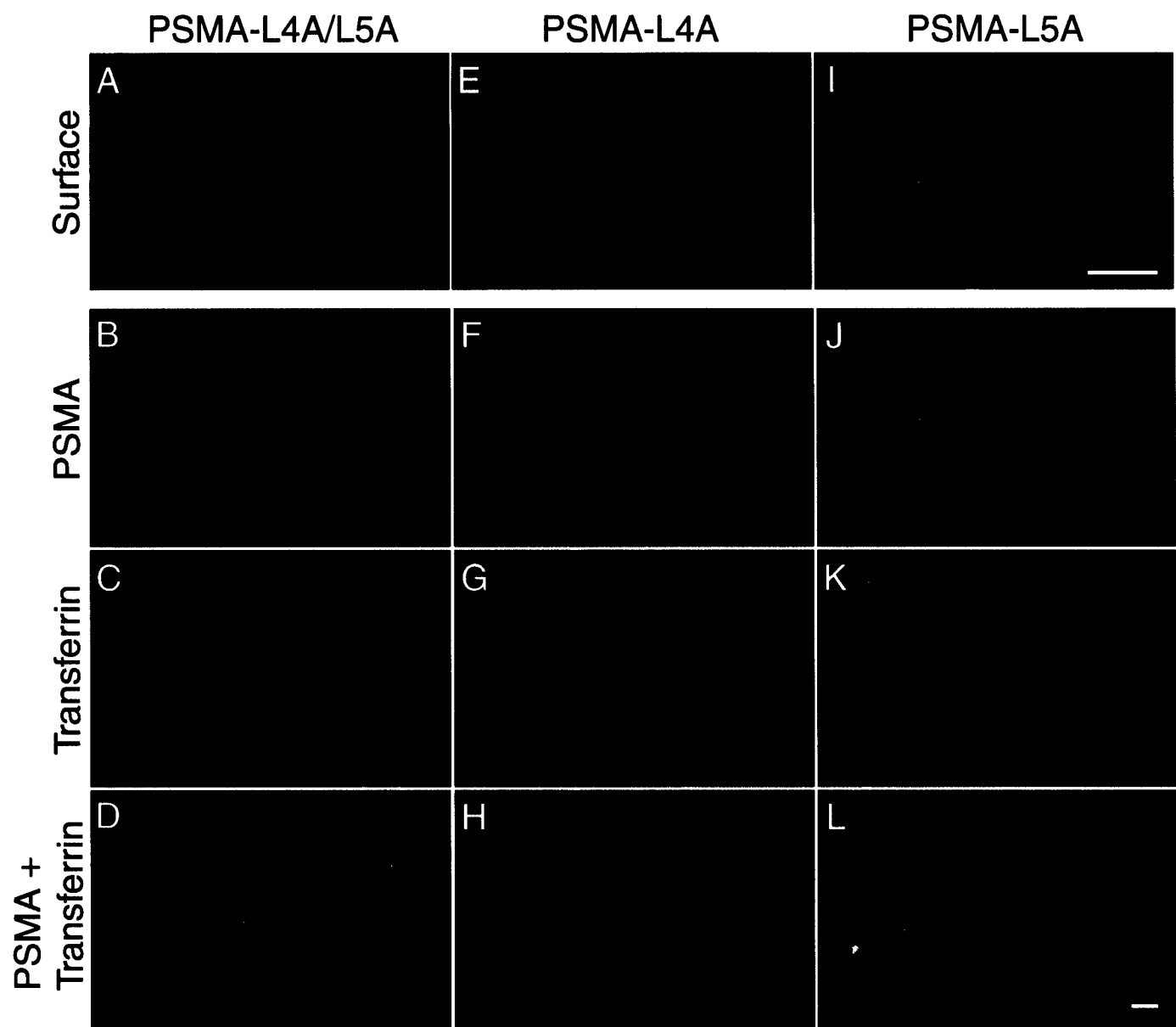


Figure 4

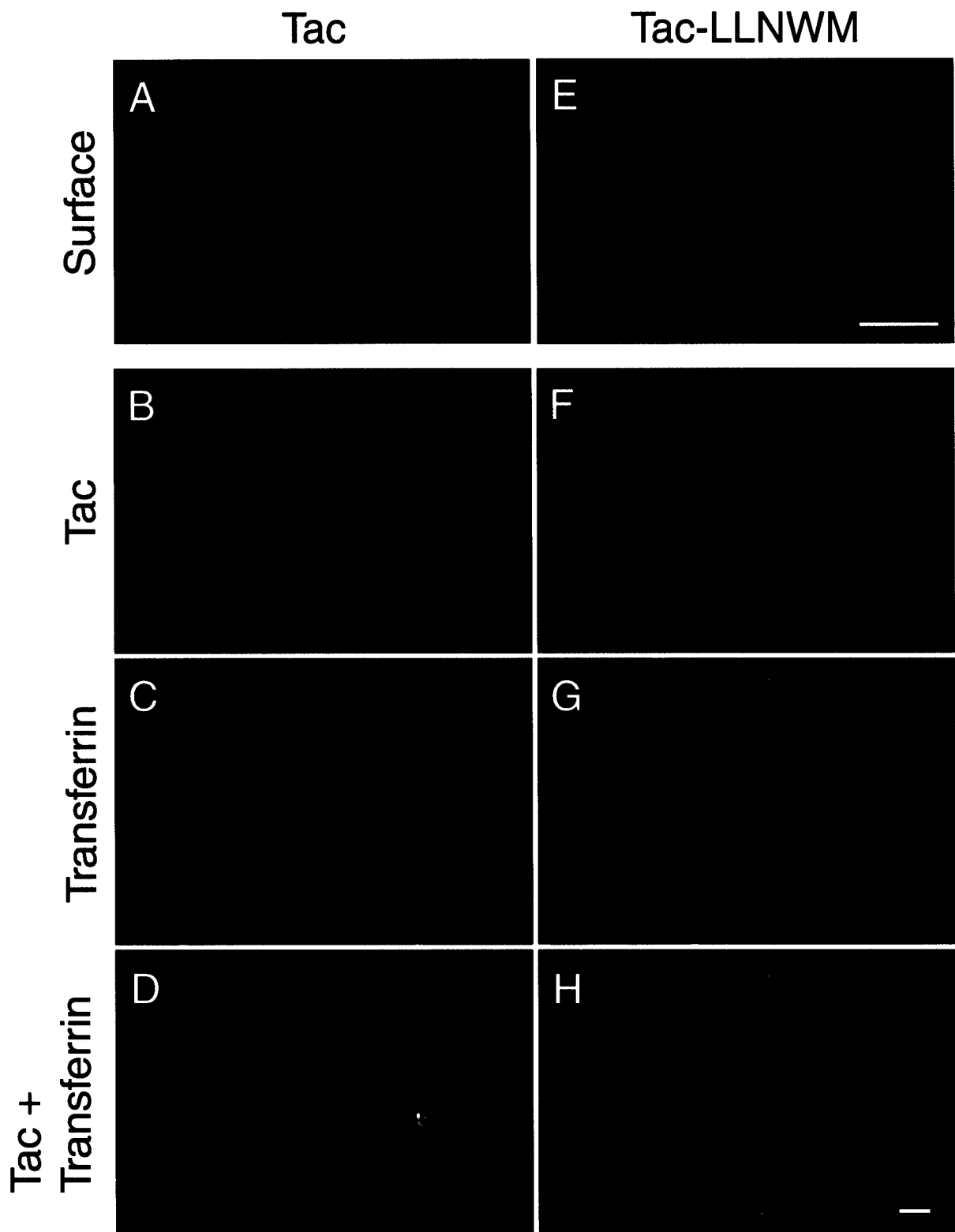


Figure 5

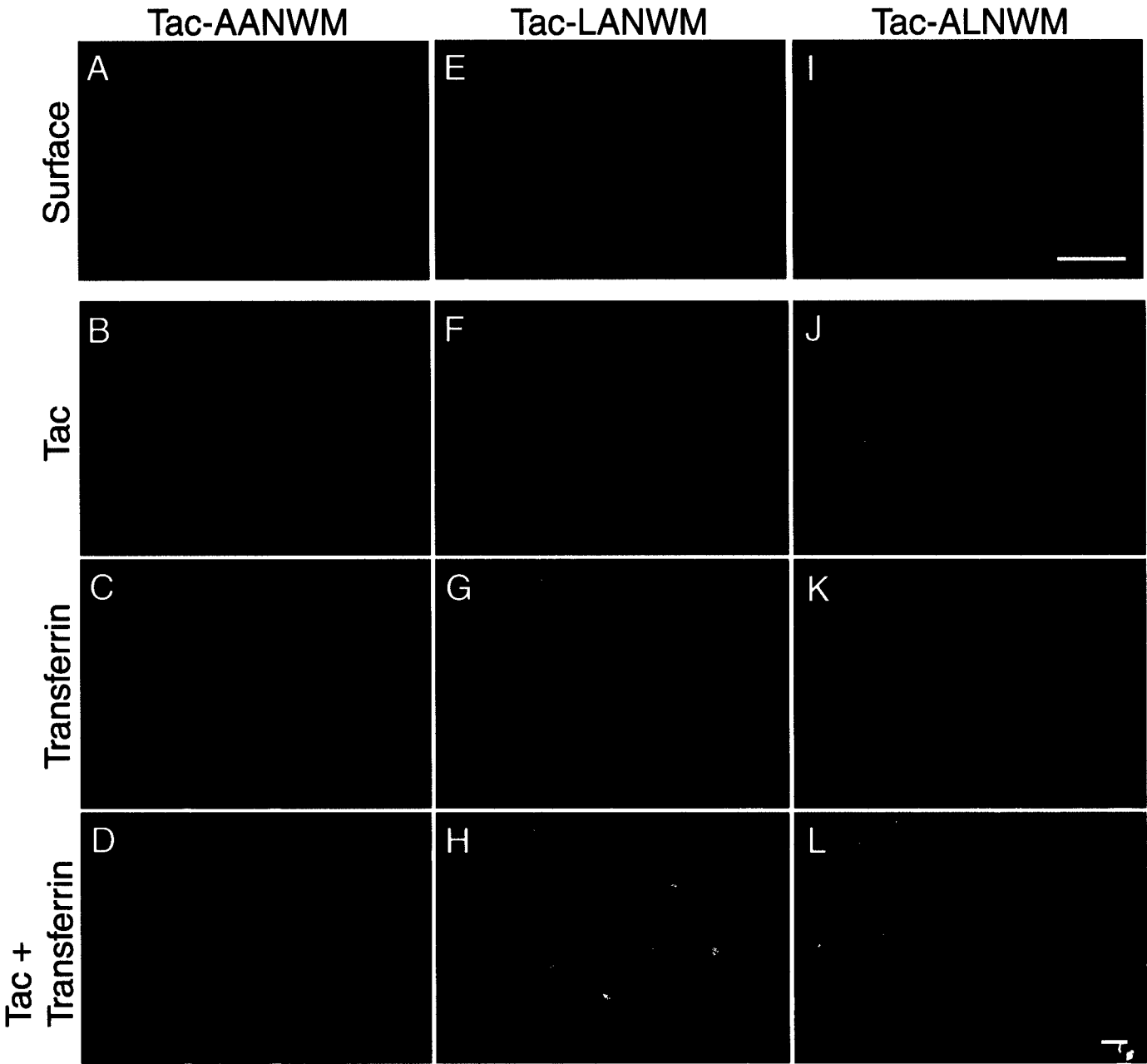
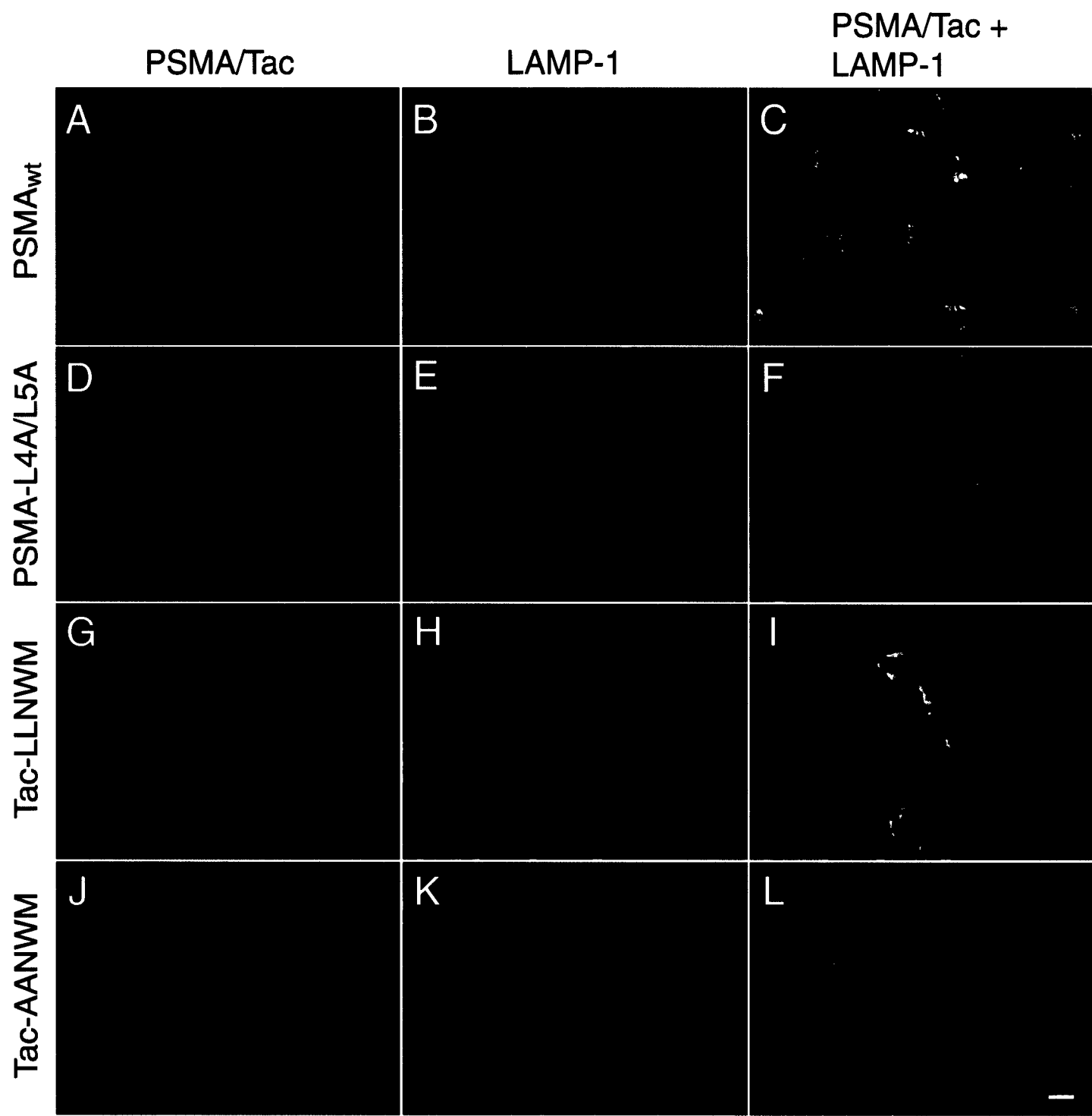


Figure 6





DEPARTMENT OF THE ARMY  
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REPLY TO  
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1 Apr 03

MEMORANDUM FOR Administrator, Defense Technical Information  
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FOR THE COMMANDER:

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PHYLLIS M. RINEHART  
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